ANTIDIABETIC ACTIVITY OF *PHALERIA MACROCARPA* (Scheff.) Boerl FRUIT EXTRACTS

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UNIVERSITI SAINS MALAYSIA
2012

ANTIDIABETIC ACTIVITY OF *PHALERIA MACROCARPA* (Scheff.) Boerl FRUIT EXTRACTS

By

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Thesis submitted in fulfillment of the requirements for the degree of Master of Science

UNIVERSTI SAINS MALAYSIA

Jun 2012

In the Name of ALLAH

The Most Beneficent and the Most Merciful

THIS THESIS IS DEDICATED

To

MY FATHER, MOTHER

BROTHERS, SISTERS, GRANTMOTHER, NEPHEWS, NIECES AND FRIENDS

ACKNOWLEDGEMENTS

Praise be to Allah, The Most Gracious, The Most Merciful, it is only by His Blessing that I could finish this work. I dedicate this work to my beloved late brother Mr. Ali who encouraged me in everlasting studies.

I would like to express my deepest sense of gratitude to my supervisor Prof. Mohamad Zaini Asmawi, for his patient guidance, encouragement and excellent advice throughout this study. My heartfelt to gratitude and appreciation goes to my co-supervisor Dr. Roziahanim Mahmud for her endless effort in ensuring this work has achieved the desired standard.

I would like to express my gratitude to the Dean of School of Pharmaceutical Sciences Assoc. Prof. Syed Azhar Syed Sulaiman, for giving me the chance to pursue my higher education in this school.

Gratitude and sincere thanks and appreciation to the dearest and best friend Safia Amer and her husband for advising, encouragement and helping

Special thanks goes to Mr. Omar Saad Ebrika and his family, Dr. Mohammed Hassan Abd Alkareem, Dr. Item J. Atangwho, Dr. Yam Mum Fei, Dr. Elsnoussi Ali Hassan, Mr. Ali Jimale, Navneet Kaur, Naziha Abdelati and her family and Rabha Omran and her family for helping, encouragement, supporting and advising all the time.

Like to thank my colleagues in the lab, Noradlin, Sookyee, Elham, Rukhsana, Yani, Rabeia and Razia, I have spent nice time with them, I also express my appreciation to the lab assistant Mr. Rosli Hassan for his excellent technical assistance.

Last but not the least; my warmest thanks are due to my family: father, mother, brothers, sisters, grandmother, nephews and nieces for helping, encouragement and prayer all the time for me.

Those who are not mentioned here, you are never forgotten, I thank you all.

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LIST OF ABBREVITIONS

^0C	Degree Celsius
A	Alpha
%	Percent
AF	Aqueous fraction
ANOVA	Analysis of variance
ARSC	Animal Research and Service Centre
AUC	Area under the curve
ATP	Adenosine triphosphate
AMP	Adenosine monophosphate
B.W.	Body weight
CF	Chloroform fraction
EAF	Ethyl acetate fraction
et al.	And others
G	Gram
H_2O_2	Hydrogen peroxide
IDDM	Insulin dependet diabetes mellitus
IC_{50}	Half maximal inhibitory concentration
i.p.	Intraperitoneal
IPGTT	Intraperitoneal glucose tolerance test
Kg	Kilogram

L	Liter
LC-MS	Liquid chromatography-mass spectrophotometer
M	Meter
Mg	Milligram
MF	Mangiferin
ME	Methanol extract
mM	Millimolar
NIDDM	Non insulin dependet diabetes mellitus
NBF	*n*-butanol fraction
NP/PEG	Natural product –polyethyene glycol
OHA	oral hypoglceamic agent
PBG	Peak blood glucose
SEM	Standard error of mean
SD	Sprague Dawley
SFI	Sub-fraction I
STZ	Streptozotocin
SSPS	Statistical package for the social sciences
TLC	Thin layer chromatography
µg	Microgram
µL	Microliter
UV	Ultraviolet
WHO	World Health Organisation
w/v	Weight over volume
w/w	Weight over volume

AKTIVITI ANTIDIABETIK EKSTRAK BUAH *PHALERIA MACROCARPA* (Scheff.) Boerl

ABSTRAK

Buah *Phaleria macrocarpa* digunakan dalam perubatan tradisional di Indonesia dan bahagian timur Malaysia sebagai ubat untuk pengurusan diabetes mellitus. Oleh itu, kajian ini dijalankan untuk mengkaji aktiviti antidiabetik buah-buahan ini dalam tikus normal dan tikus diabetik aruhan streptozotocin (STZ). Buah-buahan kering yang dikisar lumat diekstrak secara berturutan dalam petroleum eter, metanol dan air. Kesemua ekstrak (1 g/kg) tidak menunjukkan kesan terhadap glukosa darah tikus normal. Ekstrak metanol dan air merencat secara signifikan kenaikan glukosa darah tikus normal yang diberi beban glukosa dalam ujian toleransi glukosa intra-peritoneal (IPGTT). Selepas rawatan oral harian selama 12 hari, hanya ekstrak metanol (ME) menurunkan paras glukosa darah ($P<0.05$) tikus diabetik. Ekstrak metanol kemudian disisihkan untuk mendapatkan fraksi klorofom (CF), etil asetat (EAF), n-butanol (NBF) dan akueus (AF). Dalam IPGTT, NBF merencat secara signifikan ($P<0.05$) kenaikan paras glukosa darah selepas diberi beban glukosa dalam tikus normal. Rawatan oral harian dengan NBF selama 12 hari juga menurunkan paras glukosa darah ($P<0.05$) tikus diabetik. Oleh itu, NBF seterusnya disisihkan kepada sub-fraksi I (SFI) dan sub-fraksi II (SFII). SFI merencat secara signifikan ($P<0.05$) kenaikan glukosa darah dalam IPGTT dan menurunkan insulin plasma ($P<0.05$) bersama-sama dengan glukosa dalam tikus diabetik. NBF menunjukkan aktiviti perencatan yang tertinggi terhadap α-glukosidase (75%) dan α-amilase (87%) secara *in vitro*. *In vivo*, dalam tikus diabetik, NBF dan SFI juga didapati berupaya menindas puncak glukosa darah (PBG) sebanyak 15.08% dan 6.46%, masing-masing, menyebabkan penurunan dalam kawasan di bawah keluk

(AUC) sebanyak 14.23% dan 12.46%, masing-masing, selepas cabaran sukrosa oral. Kesan penindasan terhadap PBG dan AUC juga dilihat dalam ujian toleransi kanji dan glukosa, tetapi hingga takat yang lebih rendah. Keputusan ini mencadangkan perencatan oleh enzim pencernaan α-glukosidase dan α-amilase mungkin menyumbang kepada aktiviti antidiabetik *P. macrocarpa*. Penyaringan fitokimia menunjukkan kehadiran flavonoid, terpena dan tanin di dalam ME, NBF dan SFI. Analisis LC-MS mendedahkan kehadiran mangiferin dalam ME (9.52%), NBF (33.30%) dan SFI (22.5 %). Kesan *in vivo* dan *in vitro* bagi ekstrak dan fraksi-fraksi ini kelihatan berkolerasi dengan kandungan mangiferin yang mencadangkan bahawa mangiferin mungkin bertanggungiawab untuk kesan antidiabetik yang diperhatikan.

ANTIDIABETIC ACTIVITY OF *PHALERIA MACROCARPA* (Scheff.) Boerl FRUIT EXTRACTS

ABSTRACT

The fruit of *Phaleria macrocarpa* is used in traditional medicine in Indonesia and the eastern part of Malaysia, as a remedy for the management of diabetes mellitus. This study was therefore carried out to investigate the antidiabetic activity of this fruit in normal and streptozotocin (STZ) -induced diabetic rats. The pulverized dried fruits were sequentially extracted in petroleum ether, methanol and water. None of the extracts (1 g/kg) exerted an effect on blood glucose of normal rats. The methanol and water extracts significantly inhibited the rise of blood glucose after glucose loading in intra-peritoneal glucose tolerance tests (IPGTT) in normal rats. After 12 days daily of oral treatment, only methanol extract (ME), lowered the blood glucose ($P<0.05$) level of diabetic rats. The methanol extract was then fractionated to obtain chloroform (CF), ethyl acetate (EAF), *n*-butanol (NBF) and aqueous (AF) fractions. In IPGTT, NBF significantly inhibited the rise of blood glucose levels ($P<0.05$) after glucose loading in normal rats. Daily oral treatment of NBF for 12 days also lowered blood glucose level ($P<0.05$) of diabetic rats. NBF was therefore further fractionated into sub-fractions I (SFI) and sub-fraction II (SFII). SFI significantly inhibited the rise of blood glucose ($P<0.05$) in IPGTT and lowered plasma insulin ($P<0.05$) along with glucose in diabetic rats. NBF showed the highest inhibitory activity against α-glucosidase (75%) and α-amylase (87%) *in vitro*. *In vivo*, in diabetic rats, NBF and SFI were also found to suppress peak blood glucose (PBG) by 15.08% and 6.46%, respectively, resulting in a reduction in the area under the curve (AUC) by 14.23% and 12.46%, respectively, after an oral sucrose challenge ($P<0.05$). The suppressive effects on PBG and AUC were also

demonstrated in glucose and starch tolerance test, but to a lesser extent. It suggests that the inhibition by digestive enzymes α-glucosidase and α-amylase may have contributed to the antidiabetic activity of *P. macrocarpa* fruit. Phytochemical screening showed the presence of flavonoids, terpenoids and tannins, in ME, NBF and SFI. LC-MS analyses revealed the presence of mangiferin in ME (9.52%), NBF (33.30%) and SFI (22.50%). The *in vivo* and *in vitro* effect of these extract and fractions seems to be correlated with their mangiferin content which suggests that mangiferin may be responsible for the observed antidiabetic effects.

CHAPTER ONE: INTRODUCTION

1.1. Background

Daily meals should always contain a combination of carbohydrate, protein, fat, fiber, vitamins and minerals from bread, meat, oil, vegetables and fruits. The digestion process starts with amylase in the mouth, through the oesophagus and finally into the intestine. In the intestine, digested food converted into building blocks namely glucose, amino acids and fatty acids which are absorbable by the intestine. Glucose is a universal source of energy, especially for brain cells. As blood glucose reaches the postprandial level, a signal automatically triggers the pancreas to release the hormone insulin into the blood-stream. At this point, cells can utilise the glucose which is readily available. Any excessive glucose is stored in liver and muscle cells as polymeric glycogen with the help of insulin as well. This stored glucose can be consumed when it is required. It is clear how important insulin is to the body, especially for glucose control. The presence of glucose may not be recognized should insulin fail to work properly; this condition could lead to hyperglycaemia in the blood and an insufficient supply to the cells. Consequently, cells keep converting alternative sources of energy such as fat from lipid-rich tissues and protein from muscles into glucose. This alternative metabolism could lead to body weight loss and fatigue (Sonksen *et al*., 2003).

1.2. Diabetes Mellitus

Diabetes mellitus is one of the most common chronic diseases in nearly all countries. The number increases significantly as a consequence of lifestyle changes involving the lack of physical activities and indirectly mirrors the increase in obesity (Shaw *et al.*, 2010). Diabetes is classified into two main types by the underlying causes,

which are type I (insulin dependent diabetes mellitus, IDDM) and type II (non-insulin dependent diabetes mellitus, NIDDM). Anti-diabetic medications treat diabetes mellitus by lowering glucose levels in the blood. There are different classes of anti-diabetic drugs and the selection depends on the nature of the diabetes, age and the patient's condition as well as some other related factors.

IDDM occurs mostly in children and young adults, who comprise 20% of diabetics. In this condition, there is no option other than treatment with insulin's injection; insulin is the sole choice to control IDDM. The hormone needs to be injected subcutaneously since it would be destroyed if taken orally. In contrast to IDDM, patients with NIDDM would have higher levels of insulin; this is the reason why NIDDM patients are diagnosed later than IDDM patients, usually after symptoms or complications have appeared. However, NIDDM usually affects only adults, especially the obese, since obesity enhances peripheral insulin resistance. Oral hypoglycaemic agents that induce more insulin production, sensitize tissue towards insulin and prevent excessive glucose absorption are available for NIDDM patients. Two main classes used are sulfonylureas (e.g. glibenclamide) and biguanides (e.g. metformin) that can be taken individually or in combination. Obese patients are advised to take biguanides as sulfonylureas causes weight gain (Sonksen *et al.*, 1998). The number of people with diabetes is expected to increase from 1,846,000 in 2010 to 3,254,994 in 2030, and the adjusted prevalence of diabetes (adjusted to world population) in Malaysia will rise from 11.6% in 2010 to 13.8% in 2030 (International Diabetes Federation, 2009).

1.3. Pancreas

Pancreas could always be considered as a mixed gland due to its multi-functions capability. It can function as an exocrine and endocrine gland. As an exocrine gland, it acts as a digestive organ by which it secretes the digestive enzymes as well as alkaline materials and channels these into the small intestine via duct. As an endocrine gland in it secretes hormones into the blood-stream. However, the endocrine area makes up only 1% out of the total weight of the pancreas (Gerard *et al*., 1999).

Islets consist of four special groups of cells namely alpha (α) cells that produce glucagon to increase the blood glucose level, beta (β) cells that produces insulin to lower the glucose level, delta (δ) cells that secretes somatostatin and hypothalamic inhibiting hormone which also inhibits the secretion of glucagon and insulin, and finally F cells which secretes pancreatic polypeptides into the bloodstream and regulates the release of pancreatic digestive enzymes. Insulin would acts together with glucagon to regulate glucose metabolism (Donna *et al*., 1995).

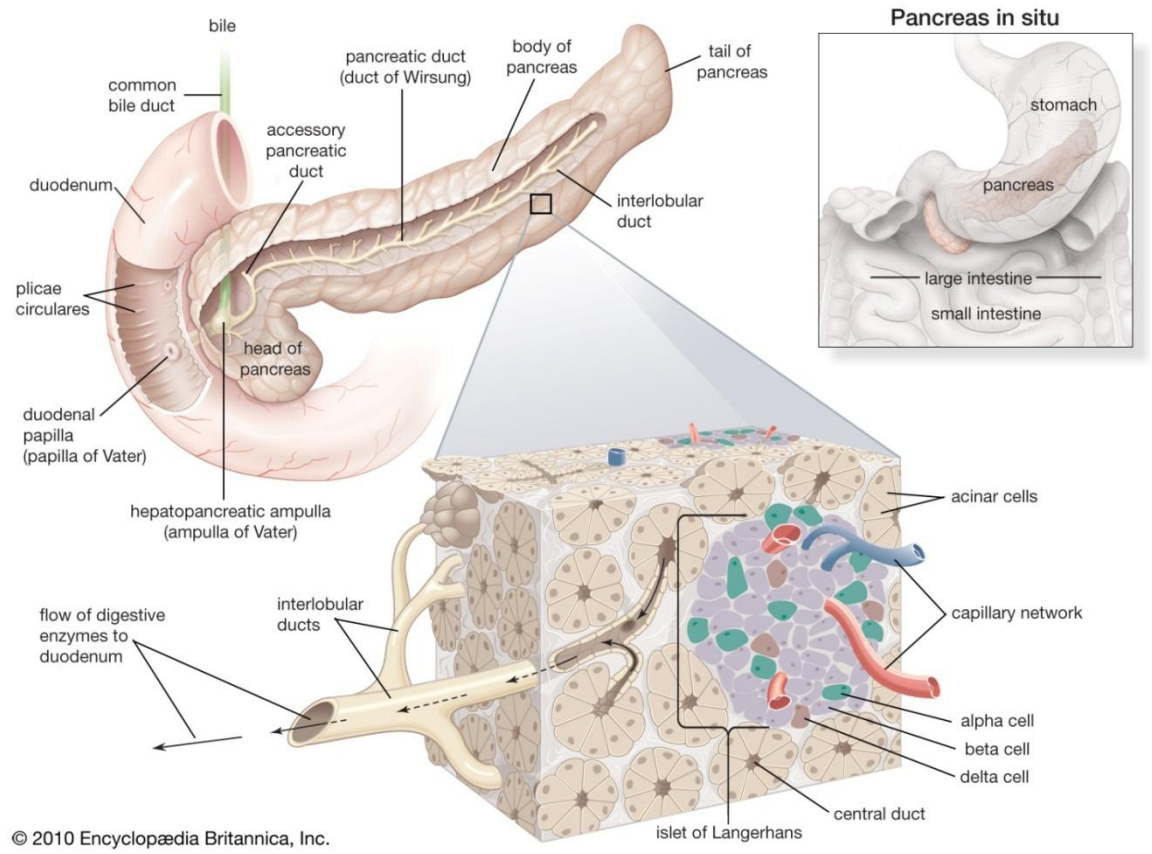

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Figure 1.1. Pancreas system

1.3.1. Glucagon

Briefly, glucagon consists of 29 amino acids (polypeptide hormone) from the α-islet cells which acts as a physiological regulator of insulin effect in response to hypoglycaemia (Laurence and Bennett, 1995). As the blood glucose level falls, it stimulates the liver to convert glycogen into glucose (glycogenolysis) so as to increase the glucose concentration. In this case, glucagon also stimulates the glycogenolysis, a process which converts non-carbohydrate sources (amino acids, lactic acid etc.) into glucose. It also stimulates fatty acids and glycerol release from adipose tissue. Apart from that, glucagon also helps to increase the concentration of AMP cyclic derived from

ATP in liver cells by activating the phosphorylase enzyme. This enzyme separates glucose units from a larger form of the glycogen molecule and boosts the free glucose units entering the bloodstream. Thus, during fasting or any time that the blood glucose level drops below normal (70-110 mg/dl), glucagon acts to increase the glucose level to normal. For this reason, it is considered as a hyperglycaemic factor (Donna *et al*., 1995).

1.3.2. Insulin

Insulin is synthesized as a large peptide (35 amino acids) containing A and B chains with an additional sequence of 16 amino acids connected as pre-proinsulin. These 16 amino acids cleaved to form proinsulin which is then hydrolysed to insulin (connected to peptide in humans) which is called the C-peptide (31 amino acids). Then, this links a threonine residue in the B chains to a glycine in the A chain (Joseph and Digregorio, 1990). β-islets cells-synthesize, release and store insulin with daily secretion in the range of 30-40 units which is equivalent to 25% of total pancreatic insulin content. A high blood glucose concentration is the principal factor that evokes insulin secretion (Laurence and Bennett, 1995).

1.4. Oral anti-diabetic drugs

The β-cell has emerged as one of the most attractive molecules that could provide a number of potential new targets in drugs development. Intensive treatment of IDDM is designed to prevent the development of micro-vascular and neurological complications, which are most likely also applicable for NIDDM (Ohkubo *et al*., 1995). However, treatment for NIDDM aims to mitigate symptoms via normalisation or near-normalisation of fasting and postprandial blood glucose levels as well as to prevent acute or long-term complications. There are several approaches to improving glucose homeostasis; however those that are currently applied in clinical practice are either unsuccessful in restoring normoglycaemia or fail after a period of time. The classes of

drugs that are readily available for glycaemic regulation are Suphonylureas (e.g. glibenclamide), Biguanides (e.g. metformin), Thiazolidinediones (e.g. pioglitazone), α-glucosidase inhibitors (e.g. acarbose), Dipeptidyl Peptidase (DPP)-IV Inhibitor and Insulin. Each of them differs by mode and site of action. These standard pharmacological treatments might be applied either individually or in combination, and provide more ideal glycaemic control in selected and identified patients (Gerard *et al*., 1999).

Table 1.1. Oral diabetes medications summary (Eurich, *et al*., 2007)

Diabetes Pills	How to Take	How They Work	Side Effects
Biguanides Metformin (Glucophage) Metformin liquid (Riomet) Metformin extended release (Glucophage XR, Fortamet, Glumetza)	**Metformin:** usually taken twice a day with breakfast and evening meal. **Metformin extended release:** usually taken once a day in the morning.	Decreases amount of glucose released from liver.	Bloating, gas, diarrhea, upset stomach, loss of appetite (usually within the first few weeks of starting). Take with food to minimize symptoms. Metformin is not likely to cause low blood glucose. In rare cases, lactic acidosis may occur in people with abnormal kidney or liver function.
Sulfonylureas Glimepiride (Amaryl) Glyburide (Diabeta, Micronase) Glipizide (Glucotrol, Glucotrol XL) Micronized glyburide (Glynase)	Take with a meal once or twice a day.	Stimulates the pancreas to release more insulin, both right after a meal and then over several hours	Low blood glucose, occasional skin rash, irritability, upset stomach

Meglitinides Repaglinide (Prandin) D-Phenylalanine Derivatives Nateglinide (Starlix)	Both of these medications should be taken with meals. If you skip a meal, skip the dose	Stimulate the pancreas to release more insulin right after a meal.	Effects diminish quickly and they must be taken with each meal; may cause low blood glucose.
Thiazolidinediones Pioglitazone (TZDs) Pioglitazone (Actos)	Usually taken once a day; take at the same time each day.	Makes the body more sensitive to the effects of insulin.	May cause side effects such as swelling (edema) or fluid retention. Do not cause low blood sugar when used alone. Increased risk of congestive heart failure in those at risk.
DPP-4 Inhibitors Sitagliptin (Januvia) Saxagliptin (Onglyza) Linagliptin(Tradjenta)	Take once a day at the same time each day	Improves insulin level after a meal and lowers the amount of glucose made by your body	Stomach discomfort, diarrhea, sore throat, stuffy nose, upper respiratory infection. Do not cause low blood glucose.
Alpha-glucosidase Inhibitors Acarbose (Precose) Miglitol (Glyset)	Take with first bite of the meal; if not eating, do not take.	Slows the absorption of carbohydrate into your bloodstream after eating.	Gas, diarrhea, upset stomach, abdominal pain
Bile Acid Sequestrants Colesevelam (Welchol)	Take once or twice a day with a meal and liquid.	Works with other diabetes medications to lower blood glucose.	Constipation, nausea, diarrhea, gas, heartburn, headache (may interact with glyburide, levothyroxine and contraceptives)

1.5. Medicinal Plants

Plants have been an exemplary source of medicine since ancient times. They have played key roles in traditional health care systems and, on the basis of this, have formed a significant percentage of allopathic and modern drugs in many nations of the world (Grover *et al*., 2002; Samy and Gopalakrishnakone, 2007). Be that as it may, to a large extent, these plants have not been valued by modern science, until recently when it became clear from empirical evidence in traditional medicine, that most pathological conditions are understood only in part, and can be traditionally managed with unimaginable efficacy, thereby warranting the current herbal renaissance going on around the world which is highly powered by the environmental drive to go green (Joy *et al*., 1998). Medicinal plants are therefore gaining reputation for use as modern alternatives to orthodox medicines or as complementary products to maintain health or treat aspects of diseases, particularly those in which orthodox medication has had limited success (Houghton, 2009).

Diabetes is one such disease that has been managed with only limited success by "Western" medicine. Conventional efforts aimed at improved management of this disease have been disappointing and the control of blood glucose level remains unsatisfactory, as is reflected in steady increases in diabetes morbidity and mortality rates (Shaw *et al*., 2010). Consequently, the current prospective focus for appropriate antidiabetic agents is herbal medicine. There is however, a need for in-depth investigation to confirm and advocate the excellence of these plants over existing therapies, such as elucidation of their mechanism(s) of action and therapeutic effects, as the antidiabetic evidence of some of them is anecdotal (Jelodar *et al*., 2007).

1.6. Research on Herbs

With the massive growth and high demand in the global market for herbal remedies, this field is estimated to grow up to USD 5 trillion in 2050 from USD 80 billion in 2000 with projected growth of USD 20 billion in 2008 (Norawi, 2002). This has boosted interest in conducting herbal research to fulfill demands. This includes plants bioactivity screening based on the inputs and claims of folklore. The WHO of Regional Office for the Western Pacific has come up with a research guideline in evaluating the safety and efficacy of herbal medicines (WHO, 1992). It gives special attention to the concept of polypharmacy in herbal preparations where the isolation of a single active substance with therapeutic value may not be necessary. However, the isolation of an active substance will remain useful in providing an exact dosage and determining any adverse reaction to the active substance (WHO, 1992).

1.7. *Phaleria macrocarpa* (Scheff.) Boerl

Kingdom : Plantae

Subkingdom : Tracheobionta

Super Division : Spermatophyta

Division : Magnoliphyta

Class : Magnolipsida

Subclass : Rosid

Order : Myrtales

Family : Thymelaeaceae

Genus : *Phaleria*

Species : *Phaleria Macrocarpa* (Scheff) Boerl

Phaleria macrocarpa (Scheff) Boerl is a shrub or small tree; usually up to 5 meters (Winarto, 2003; Harmanto, 2005) and the height sometimes could also reach up to 18 meters. It features many branched crown, a large straight root (one meter long) exuding sap, a brownish green bark and white wood. It has green tapering leaves, 7-10 cm length and 3-5 cm width. Its flowers form a compound of 2-4 flowers, little white trumpet - like fragrant flowers. The fruit comes in various sizes and the color varying from green to maroon. The pit is in a round shape, white in color and very poisonous. It grows in areas of 10–1200 m above the sea level, and best in areas of 1000 m above the sea level with its productive age estimated to be in between 10-20 years.

For centuries, the native Indonesians have used the fruits and leaves of the Mahkota Dewa (literally translated as God's Crown) tree, *P. macrocarpa* (Scheff.) Boerl., to combat diabetes, liver diseases, vascular problems, cancer, and high blood pressure.

Recent research has proven that *P. macrocarpa* contains plant secondary metabolites that could combat not only cancers or infectious disease such as malaria, but also the so-called lifestyle diseases (Harmanto, 2005). Parts of *P. macrocarpa* that are used for medical treatments are the stems, leaves and fruits. Empirically, *P. macrocarpa* is proven capable of controlling cancer, impotency, dysentery, hemorrhoid, diabetes mellitus, allergies, liver and heart diseases, kidney disorders, blood diseases, arthritis, rheumatism, high blood pressure, stroke, migraine, various skin diseases, acne and the level of cholesterol. This plant contains antihistamine, antioxidant and anti-cancer substances (Harmanto, 2005).

Kusmardiyani and co-workers (2004) isolated a white yellowish and odorless crystalline compound from the ethanolic extract of *P. macrocarpa* leaves. The isolate was presumed as a benzophenone glucoside based on its ultraviolet, infrared, mass, 1H and 13C NMR spectra. Another benzophenoic glucoside known as 4,5-dihydroxy,4'-methoxybenzophenone-3-O-ß-D-glucoside or Phalerin was also isolated from the methanolic extract of the leaves of *P. macrocarpa*. Phalerin was cytotoxic to myeloma cell line (NS-1) having IC50 of 83µg/ml or 1.9x10-1 mM (Mae, *et al*., 2005).

A lignan which is similar to syringaresinol was isolated by Lisdawati (2002) from the ethyl acetate fraction of P. macrocarpa's mesocarp. The isolate was elucidated by using ultraviolet – visible spectroscopy (UV-Vis) spectra data, Fourier transform infra red (FTIR) spectrometric, liquid chromatography–mass spectrometry (LC-MS), and the proton nuclear magnetic resonance spectral data 1H–RMI and combination of 2D 1H,1H-COSY, TOCSY and NOESY RMI. The spectral evidence show molecule structures of the isolate was C19H20O6: 5-[4(4-Methoxy-phenyl)-tetrahydrofuro[3,4-c] furan-1-yl]-benzene- 1,2,3-triol. Besides, *P. macrocarpa* too contains gallic acid which can exhibit a significant inhibition of cell proliferation in a series of cancer cells and

induction of apoptosis in esophageal cancer cell but not in non - cancerous cell (Faried, *et al*., 2007).

Hendra, *et al*., (2011) reported that methanol extract of various parts of *P. macrocarpa* could be considered as a natural antimicrobial source due to the presence of flavonoid compounds. Flavonoid compound present in *P. macrocarpa* fruits were analyzed by using Reversed Phase-High Performance Liquid Chromatography (RP-HPLC). Kaempferol, myricetin, naringin, and rutin were found as flavonoid compounds in pericarp of *P. macrocarpa*. It was also confirmed the presence of naringin and quercetin in mesocarp. Apart from that, the seed of *P. macrocarpa* fruits were reported only contain quercetin.

Triastuti, *et al*., (2008) reported that the ethyl acetate of *P. macrocarpa* had diabetic activity against alloxan - induced diabetic rats which is mediated either by preventing the decline of hepatic antioxidant status or due to its indirect radical scavenging capacity. Triastuti, *et al*., (2009) have also reported that the methanol extract and its fraction of *P. macrocarpa* consist of anti-hyperglycemic and anti - nephropathy of *P. macrocarpa*. This may be correlated to the increase of renal antioxidant enzyme activity in alloxan - induced diabetic rats.

Sugiwati, *et al*., (2006) reported that the n-butanol of young and ripe fruit extracts of *P. macrocarpa* contain the highest inhibitory activity followed by ethyl acetate and methanol extracts of in vitro α-glucosidase. By oral administration to rats, the hypoglycemic activity showed that the boiled water extract and n-butanol extracts of ripe fruit (at a certain dose) had significantly decreased the blood glucose concentration of rats after been treated with 80% w/v sugar solution, being comparable to those of acarbose rats as the positive control.

Sugiwati, *et al*., (2009) has also reported that the ethyl acetate which was extracted from the old leaves of *P. macrocarpa* has given the highest inhibition activity based on α-glucosidase inhibition test compared to the young leaves. The inhibition activity from the methanol and boiled water extracts of old leaves too were found to be greater and higher than that of young leaves. In addition,

Oshimi, S *et al*., (2007) had isolated icariside C3, phalerin and mangiferin from the fruits of *P. macrocarpa*. Based on this study, the icariside C3 showed a slow vasorelaxant activity against noradrenalin-induced contraction of isolated rats' aorta.

Sellamuthu, *et al*., (2009) reported that mangiferin purified from methanolic root extract of *Salacia chinensis* has the antihyperglyceamic activity in normal and streptocotozin-induced diabetic rats. The mangiferin was administrated orally at a dose of 40 mg/kg weight per day (30 days) to STZ-induced diabetic rats. The mangiferin treated diabetic rats significantly decreased the level of blood glucose, glycosylated hemoglobin as well as increased level of insulin and hemoglobin.

Miura, *et al*., (2001) isolated mangiferin from *Anemarrhena asphodeloides Bunge rhizome,* and tested for antidiabetic activity in KK-Ay mice, an animal model of type-2 diabetes. They found that MF lowered the blood glucose level of KK-Ay mice three weeks after oral administration.

Miura, et al., (2001) also reported that water extract of *Anemarrhena asphodeloides Bunge rhizome* (90 mg/kg) reduced blood glucose levels from 570 +/- 29 to 401 +/- 59 mg/dl 7 h after oral administration ($p<0.05$) and also tended to reduce serum insulin levels in KK-Ay mice. *Anemarrhena asphodeloides*-treated KK-Ay mice had significantly reduced blood glucose levels in an insulin tolerance test.

Iwamoto, *et al*,. (2000) reported that the antidiabetic action of mangiferin with exercise was investigated in KK-Ay mice, an animal model of type 2 diabetes. MF (30 mg/kg) significantly decreased the blood glucose and insulin levels of KK-Ay mice with exercise two weeks after the oral administration, while control group (exercise only) did not changes. MF also significantly decreased blood triglceride level of KK-Ay mice. These finding indicate that MF with exercise is useful for the early stage symptom of type 2 diabetes.

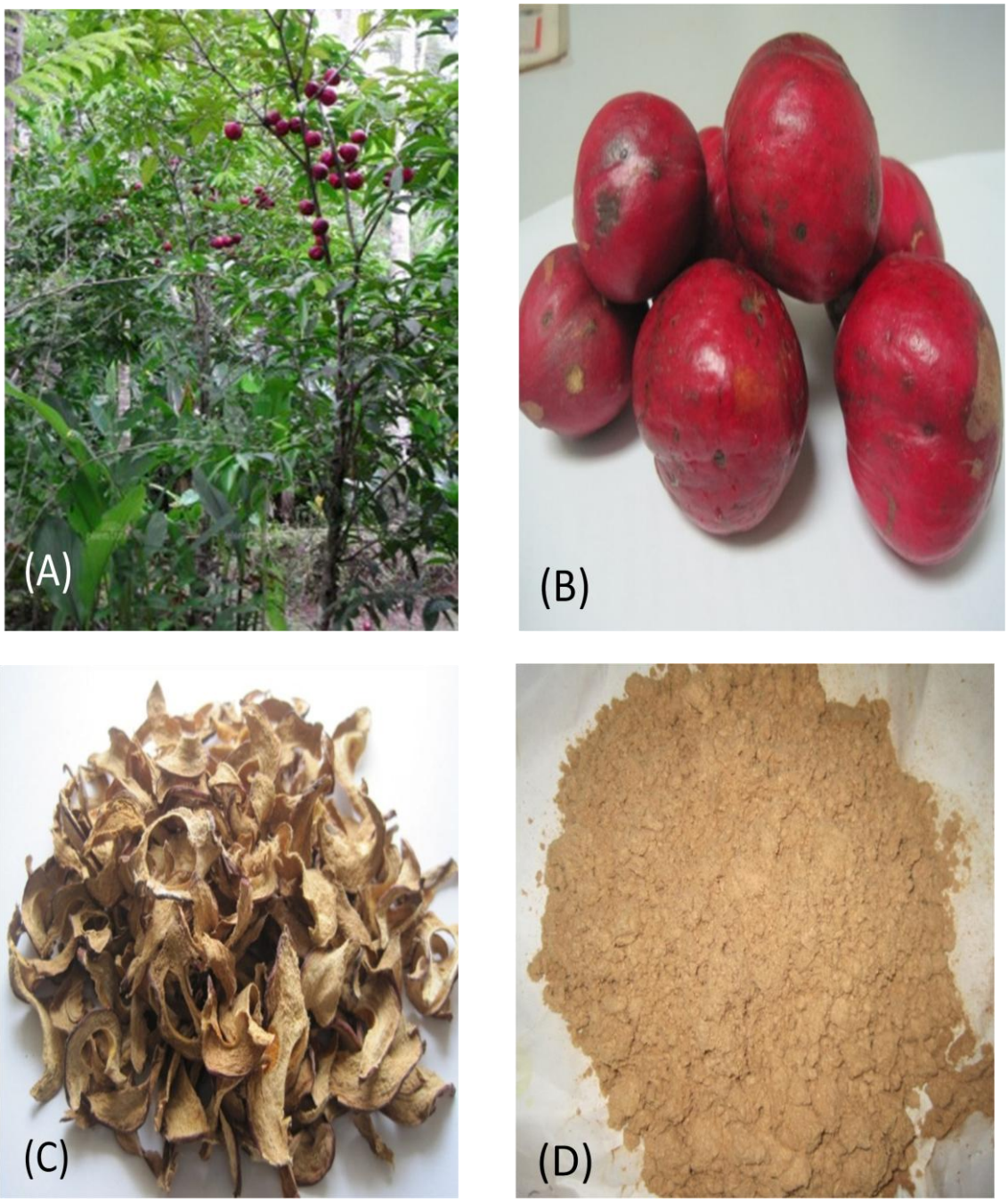

Figure 1.2. The plant *Phaleria macrocarpa* (Scheff) Boerl. (A) Whole plant. (B) Ripe fruits. (C) Dried sliced fruits. (D) Ground fruits

1.4 Research Objectives

In this study, dried, sliced and ground *P. macrocarpa* fruits were extracted and the contents as well as the antidiabetic effect of each of the extract and some fractionated samples were evaluated, as described in chapter 2. The objectives of the present study were:

1. To study the hypoglycaemic and anti-hyperglycaemic activity of the extracts using hypoglycaemic and intra-peritoneal glucose tolerance tests (IPGTT).

2. To determine the most active extract and the most active fraction in an attempt to identify the active compounds by bioactivity-guided fractionation technique.

3. To evaluate the effect of most active extract, fraction and sub-fraction on α-glucosidase and alpha amylase inhibition *in vitro* and determine the IC_{50}.

4. To investigate the effect of the most active extract, fraction and sub-fraction of *P. macrocarpa* on *in vivo* alpha glucosidase inhibition tests on normal and diabetic rats.

5. To perform a phytochemical screening study of chemical groups present in the most active extract, fraction and sub-fraction by TLC and LC-MS.

CHAPTER TWO: MATERIALS AND METHODS

2.1. Instruments used and their sources

Accu-check Advantage II Clinical Glucose meter	Roche diagnostic Co. USA
Electric grinder	Apex-mill, Comminuting mill, England
Flurescence Analysis Cabinet	Westbury, USA
Freeze dryer	Labconco Cooperation, Denmark
Freezer	Forma Scientific, USA
Hitachi U-2000 Spectrophotometer	Hitachi, Japan
Hot plate/Stirrer	PMC Industries, USA
Microplate reader	Power Wave X340, USA
Oral needle	Popper & Sons, Inc, USA
Oven	Memmert, Germany
Rotary evaporator	Buchi, Switzerland
Separatory funnel	Schott, Duran, Germany
Soxhlet-extractor	Buchi, Switzerland
TLC plate silica gel 60 F_{254}	Merck, Germany
Vacuum system	Buchi, Switzerland
Vortex mixer (Model: VM-2000)	VIH DER Instruments Co, Taiwan
Water bath	Brotech, Malyasia

2.2. Materials used and their sources

3,5-Dinitrosalicylic acid	Sigma Aldrich Chemical Co, US
Acetic acid	R & M Chem., UK
Anisaldehyde reagent	Sigma-Aldrich, Germany
Anhydrous monobasic sodium phosphate	BDH Chemical Ltd, England
Chloroform	Fisher Scientific, UK
Dibasic sodium phosphate	Sigma Aldrich Chemical Co, USA
Dragendorff reagent	Sigma-Aldrich, USA
Ethyl acetate	Fisher Scientific, UK
Formic acid	R & M Chem., UK
Glibenclamide	Novo Nordisk (Copenhagen,Denmark)
Glucose monohydrate	Essex, USA
Human insulin 100 IU/Ml	Novo Nordisk (Copenhagen,Denmark)
Mangiferin	Sigma-Aldrich, Germany
Metformin	Novo Nordisk (Copenhagen,Denmark)
Methanol [CH_3OH]	Fisher Scientific, UK
n-butanol	Fisher Scientific, UK
Natural product reagent	Sigma-Aldrich, Germany
Para – nitophenyl – α – glucopyranoside	Sigma Aldrich Chemical Co, US
Petroleum ether	Fisher Scientific, UK
Silica gel-7730	Merck, Germany
Sodium chloride	R & M Chemicals, Essex, UK
Sodium hydroxide	R & M Chemicals, Essex, UK
Sodium potassium tartrate	R & M Chemicals, Essex, UK

Starch	Ajax Chemical, Sydney, Australia
Streptozotocin	Sigma-Aldrich Chemical Co, USA
Sulphuric acid [H_2SO_4]	R & M Chem, UK
Tween 80	R & M Chem., UK
α-glucosidase from yeast	Sigma-Aldrich Chemical Co, US

2.3. Methods

2.3.1. Experimental animals

Healthy male Sprague Dawley (SD) rats weighing between 200-250g obtained from the Animal Research and Service Centre (ARASC), Universiti Sains Malaysia (USM) were used in the study. The animals were housed and kept at 25-30^{0}C in the Animal Transit Room, School of Pharmaceutical Sciences, USM. They were allowed access to food (standard laboratory chow, Gold Coin Sdn. Bhd., Malaysia) and tap water *ad libitum*. The experimental procedures were approved by the Animal Ethics Committee of Universiti Sains Malaysia (USM) Penang, Malaysia.

2.3.2. Plant material collection and preparation of extracts

The dried fruits of *P. macrocarpa* Benth were collected from Kepala Batas, Seberang Perai, Pulau Pinang, Malaysia. A voucher specimen of the plant (11259) is deposited at Herbarium of School of Biological Sciences, Universiti Sains Malaysia. These dried fruits were grounded into powder using a milling machine, and thereafter weighed and stored in air tight containers until use. A sample of 2400 g of the grounded plant was sequentially extracted with petroleum ether, then methanol using soxhlet apparatus (40^{0}C) for 48 hours each. The residue from methanol extraction after complete drying was re-extracted with water by maceration at 60^{0}C for 24 hours as shown in Figure 2.1. Each extraction step was repeated three times and the different extracts obtained were filtered with Whatman No. 1 filter paper and concentrated *in vacuo* by rotary evaporation (Buchi, Switzerland) at reduced pressure. The concentrated extracts were frozen at -70 ^{0}C for 48 hours then freeze-dried under vacuum for 24 hours. The dried extracts were kept in the freezer from where sample were withdrawn from time to time for test procedures.

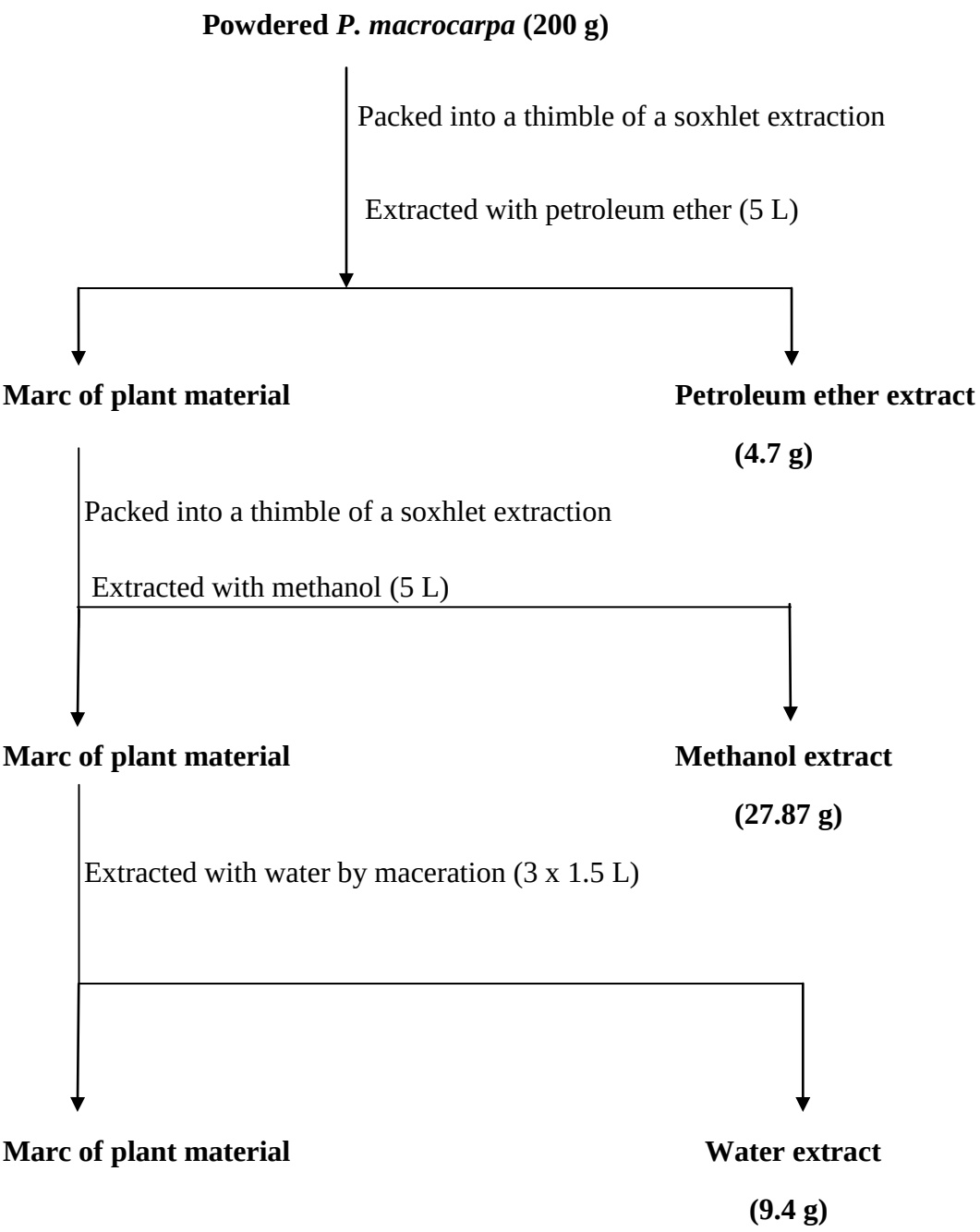

Figure 2.1.Schematic diagram of the extraction procedure of *P. macrocarpa* fruits

2.3.3. Antidiabetic evaluation of *P. macrocarpa* extracts

2.3.3.1. Hypoglycaemic activity in normal rats

Glucose response to a single dose of the extracts (1 g/kg b.w.) compared to standard antidiabetic drugs was carried out in 30 overnight fasted normal rats (200-250 g). These rats were divided equally into five groups of six rats according to the following treatment:

Group I served as a negative control and received normal saline, 10 mL/kg

Group II served as a positive control and treated with glibenclamide, 10 mg/kg

Group III received 1 g/kg b.w. of petroleum ether extract of *P. macrocarpa*

Group IV received 1g/kg b.w. of methanol extract of *P. macrocarpa*

Group V received 1 g/kg b.w. of water extract of *P. macrocarpa*

The extracts/drug/saline was administered orally via intra-gastric tube. Blood samples were collected from tail vein prior to dosing (0 hour) and then at 1, 2, 3, 5 and 7 hours after dosing, for glucose level determination using a clinical glucose meter (Accu-check Advantage II, Roche Diagnostics Co. USA).

2.3.3.2. Intra-peritoneal glucose tolerance activity (IPGTT) in normal rats

Thirty Male Sprague Dawley rats (200–250 g) were equally divided into five groups (n=6) and fasted over night. The first group received normal saline 10 ml/kg as control. The second group was treated with metformin 500 mg/kg. The third, fourth and fifth groups received 1 g/kg petroleum ether extract, methanol extract and water extract of *P. macrocarpa,* respectively. After an hour, the rats were administrated glucose (1 g/kg b.w.) intra-peritoneally and blood samples withdrawn via tail nicking at times 0 (before treatment), 15, 30, 45, 60, 90 and 120 minutes after the glucose loading for

determination of blood glucose level using Accu-check Advantage II (Roche Diagnostics Co. USA).

2.3.3.3. Anti-hyperglyceamic activity in streptozotocin - induced diabetic rats

Diabetes was induced in rats by intra-peritoneal injection of 65 mg/kg b.w. of streptozotocin (STZ) after an overnight fast folloing method described previously (Abdul-Razak *et al*., 2002). 72 hours after streptozotocin administration, blood glucose level was measured. Rats with fasting blood glucose ≥ 15 mmol/L were considered diabetic and used for the study.

Forty two (42) diabetic and 6 non diabetic rats were assigned into 8 groups of 6 rats each and treated as shown below.

Group1: 6 normal rats treated with normal saline 10 mL/kg b.w.

Group2: 6 Diabetic rats treated with normal saline 10 ml/kg b.w.

Group3: 6 Diabetic rats treated with glibenclamide 10 mg/kg b.w.

Group4: 6 Diabetic rats treated with metformin 250 mg/kg b.w.

Group5: 6 Diabetic rats treated with insulin 5 I.U./kg b.w.

Group6: 6 Diabetic rats treated with petroleum ether extract of *P.macrocarpa* 1 g/kg.

Group7: 6 Diabetic rats treated with methanol extract *P.macrocarpa* 1 g/kg b.w.

Group8: 6 Diabetic rats treated with water extract *P.macrocarpa* 1 g/kg b.w.

Treatment was once a day, and lasted for 12 days. While the drug/extracts were administered via intra-gastric oral tube, insulin was given subcutaneous. Blood glucose during treatment period (3rd, 6th, 9th days) and at end of study 12th day was monitored using Accu-check Advantage II with tail vein blood.

2.3.3.4. Dose response relationship of most active extract (methanol extract of *P. macrocarpa*)

Rats were divided into five groups and treated as follows:

Group1: Rats received normal saline 10 mL/kg as a negative control.

Group2: Rats were treated with metformin 500 mg/kg as a positive control.

Group3: Rats were treated with 250 mg/kg methanol extract of *P. macrocarpa*.

Group4: Rats were treated with 500 mg/kg methanol extract of *P. macrocarpa.*

Group5: Rats were treated with 1000 mg/kg methanol extract of *P .macrocarpa.*

After an hour, the rats were loaded with 1 g/kg glucose and blood glucose level was measured before treatment and after 15, 30, 45, 60, 90 and 120 minutes been loaded with glucose.

2.3.4. Fractionation of methanol extract by using solvent-solvent extraction method

The methanol extract of *P. macrocarpa* was further fractionated as following: the methanol extract (110 gm) was first suspended 500 mL of water. Then the suspension obtained was poured into a 1L separatory funnel. The solution was extracted with chloroform (3×250 mL). The combined chloroform fraction was dried by using anhydrous sodium sulphate, followed by solvent evaporation in a rotary evaporator.

The aqueous layer was then extracted with ethyl acetate (3×250 mL). The combined ethyl acetate fraction was washed with water, dried over anhydrous sodium sulphate and concentrated further with rotary evaporator.

Finally, the aqueous layer was extracted with *n*-butanol (5×250 mL). Then, the combined *n*-butanol fraction was concentrated using the rotary evaporator. The remainder aqueous was also concentrated in rotary evaporator. Concentrated fractions